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14. ABSTRACT

In attempts to understand how the signaling by retinoic acid (the active vitamin A metabolite) is regulated we have been studying the retinoic acid binding protein called CRABP-II. These studies revealed that CRABP-II acts to enhance the transcriptional activities of RA and that it does so by directly delivering the hormone to its cognate transcription factor, RAR. Consequently CRABP-II dramatically sensitized cultured mammary carcinoma cells to RA-induced growth inhibition. Similarly, over-expression of CRABP-II inhibited mammary tumor growth in two different mouse models of cancer. CRABP-II may be a novel target for therapeutic and preventive strategies for retinoid-treatment of breast cancer. This project aims to delineate the mechanism by which CRABP-II modulates RA activity, especially as related to its ability to enhance the anti-proliferative action of the ligand.

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Introduction

Retinoic acid (RA), the active metabolite of vitamin A, plays critical roles in embryonic development as well as growth and differentiation in adult mammals. Retinoic acid is currently used or is in clinical trials for treatment of a variety of different cancers, including breast cancer (*1*). Although retinoids are efficacious, pharmacological doses often result in toxicity (*2*). The anticarcinogenic activities of RA are mediated by the ligand-inducible transcription factors termed retinoic acid receptors (RARs) (*3*). Like other type-II nuclear receptors, RARs function as heterodimers with the retinoid X receptor (RXR). These heterodimers associate with specific DNA sequences (RAR response elements, RARE) comprised of two direct repeats of the consensus sequence PuG(G/T)TCA, separated by either 2 (DR-2) or 5 (DR-5) basepairs (*3, 4*). RXR-RAR heterodimers thus bind in regulatory regions of their target genes and enhance transcriptional rates upon binding of RA (*5*). In cells, RA also associates with cellular retinoic acid binding proteins (CRABP-I and CRABP-II). We recently showed that CRABP-II carries RA from the cytoplasm to the nucleus where it channels the ligand directly to RARα via a transient protein-protein interaction (*6, 7*). This "ligand channeling" sensitizes cells to transcriptional activation by RAR (*6*). Indeed, over-expression of the binding protein dramatically lowered the effective RA concentration necessary to induce growth inhibition in mammary carcinoma cells (*7*). Similarly, over-expressing CRABP-II inhibited mammary tumor growth in two different mouse models of cancer. The goal of this project is to elucidate the mechanisms by which CRABP-II directs RA signaling to enhance anti-proliferative responses in mammary carcinoma cells.

Body

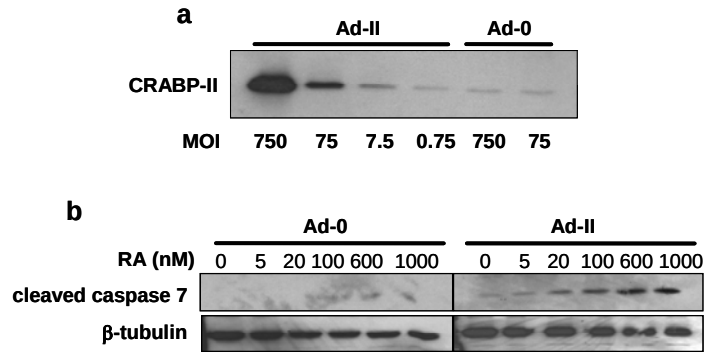
<u>To determine the mechanism by which CRABP-II inhibits growth of mammary carcinoma cells</u>.

Our group has previously shown that over-expression of CRABP-II enhances the ability of RA to inhibit the growth of the mammary carcinoma cell line MCF-7 (*7*). The goal of this project is to determine the mechanisms by which RA inhibits the growth of these cells. We tested two signaling pathways that may respond to RA through RAR-mediated transcriptional regulation to induce growth arrest: (1) <u>Apoptosis</u>- RA may induce programmed cell death, characterized by cleavage of cytosolic cysteine proteases, caspases, and by DNA fragmentation. (2) <u>Cell cycle arrest</u>- RA could induce growth arrest by blocking the cell cycle at a particular stage.

<u>RA induces apoptosis in MCF-7 cells (work published in (*8*))</u>

Studies published during this time period indicate that RA causes MCF-7 cells to undergo apoptosis after five days of treatment (*8*). This was indicated by flow cytometry studies which revealed significant RA-induced DNA fragmentation. Apoptotic responses are coordinated by a tightly regulated group of cysteine proteases named caspases. In a normal healthy cell, these proteins are found in an inactive state. Upon stimulation of apoptosis, procaspases are cleaved to produce active proteases which then cleave cellular targets to propagate the apoptotic signal and induce death. Therefore, apoptotic responses can be detected by monitoring the appearance of cleaved caspases. Treatment of MCF-7 cells with RA for 5 days caused a dose dependent activation of caspase 9 and cells that ectopically over-expressed CRABP-II displayed enhanced caspase 9 cleavage. RA also induces cleavage of caspase 7 and CRABP-II dramatically enhances this activity (Figure 1). CRABP-II was overexpressed by using an adenovirus

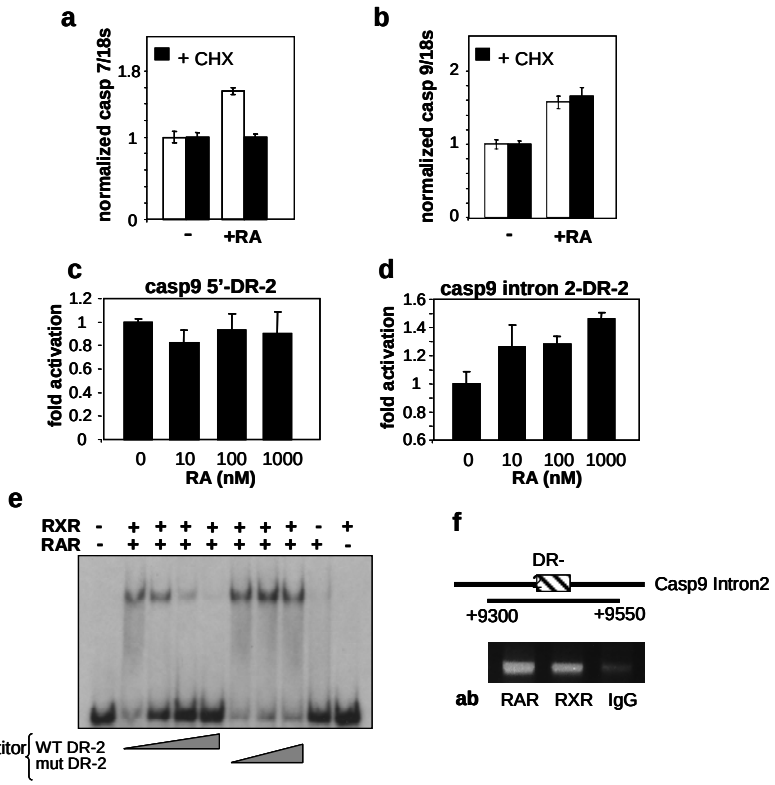
Figure 1 CRABP-II Enhances RA-Induced Caspase 7 Cleavage



encoding the gene's cDNA (Figure 1a). Caspase 7 cleavage was slightly induced upon RA treatment (Figure 1b left panel). Upon over-expression of CRABP-II, caspase 7 cleavage was greatly enhanced (Figure 1b right panel). Therefore, RA-induced apoptosis is associated with several caspase activation events including caspase 9 and caspase 7, and CRABP-II cooperates with RA in mediating the response (*8*).

To identify the genes involved in these responses, we carried out expression array studies using Affymetrix cDNA hybridization chips to identify genes that are up-regulated in response to RA. The expression levels of many genes involved in apoptotic responses were induced. Specifically, caspase 7 and caspase 9 genes were both induced in response to RA. These observations suggest a mechanism by which RA triggers the apoptotic response in MCF-7 cells. In the past year we have examined the mechanism by which these genes are induced by RA. Regulation of expression of these genes by RA may be exerted directly, i.e. mediated by an RAR response element (RARE). Alternatively, responses may reflect secondary events involving RAR control of immediate target genes, which, in turn, are involved in secondary events leading to the observed modulation. Hence, an important question that arises is which of the RA-controlled pro-apoptotic genes in MCF-7 cells comprise direct targets for RAR. This question is particularly pertinent considering the present paucity of information on the mechanisms by which RA exerts its anticarcinogenic activities. To begin to address this issue, we examined whether either caspase 7 or caspase 9 are under direct RAR control. To validate the array data, quantitative real-time PCR (Q-PCR) was carried out. MCF-7 cells were treated for 4 hr with RA (50 nM) or vehicle and Q-PCR was used to compare the expression levels of mRNA for caspase 9 and caspase 7 in RA-treated *vs*. untreated cells (Fig. 2). In good agreement with the Affymetrix array data, the expression levels of mRNA for both caspase 9 and caspase 7 increased by about 60% in response to RA treatment. To determine whether these genes are direct targets for transcriptional regulation by RA, the effect of the protein synthesis inhibitor cycloheximide on their induction by RA was studied. Cycloheximide treatment will abolish secondary events that require *de novo* protein synthesis, but will not affect direct transcriptional responses. The analyses showed that inhibition of protein synthesis completely abolished the RA-response of caspase 7 expression (Fig. 2a). In contrast, caspase 9 mRNA was up-regulated by RA regardless of the presence of cycloheximide (Fig. 2b). Hence, while the effect of RA on caspase 7 expression is a secondary response, *caspase 9* is likely to be a direct target for RAR signaling.

Figure 2 *Caspase 9* is a direct target for RAR and possesses a functional RARE in its second intron 2



Consensus RAREs are comprised of two direct repeats of the sequence PuG(G/T)TCA spaced by either 2 (DR-2) or 5 (DR-5) basepairs. We used two programs, TransFac (www.gene-regulation.com, (*9*)) and TESS (www.cbil.upenn.edu/tess, (*10*)), to screen the human *caspase 9* gene and adjacent regulatory sequences for potential RAREs. Within a stretch of eight kilobases upstream of the *caspase 9* start site (1p36.3), a potential RARE comprised of the non-canonical DR-2 sequence AGGTCAgcAGTTCG was found at position -1690. This element along with 38 basepairs of flanking sequences on both sides was cloned into a luciferase reporter

vector and its functionality was examined by transactivation assays carried out in MCF-7 cells. The expression of the reporter did not respond to RA (Fig 2c), suggesting that the element does not function as an RARE. An additional potential RARE, comprised of the consensus DR-2 sequence AGGTCAggAGTTCA was found in the second intron of the *caspase 9* gene, 9461 bp downstream of the start site. This RARE and 45 basepairs of flanking sequences on each side were cloned into a luciferase reporter which was used in transactivation assays. The data (Fig. 2d) demonstrated a dose-responsive activation of reporter expression by RA, suggesting that the element indeed comprises a functional RARE. To verify that the element can bind RAR-RXR heterodimers, electrophoretic mobility-shift assays (EMSA) were carried out. RARα and RXRα lacking their amino terminal A/B domains (RARαΔAB and RXRαΔAB) were expressed in *E. coli*, purified, and examined for binding to a 90 basepair oligonucleotide harboring the putative response element and its flanking sequences. The data (Fig. 2e) demonstrated that RAR-RXR heterodimers tightly and specifically associate with the DR-2 element of the second intron of *caspase 9*. Finally, to examine whether the element is occupied by RXR-RAR heterodimers in a living cell, chromatin immunoprecipitation (ChIP) assays were carried out. Proteins were crosslinked to chromatin in MCF-7 cells and immunoprecipitated using antibodies for RAR or RXR, or non-specific IgG. Precipitates were sonicated, the crosslink reversed, DNA isolated, and a 250 bp region surrounding the DR-2 in the second intron of *caspase 9* amplified by PCR. The data (Fig. 2f) showed that antibodies against either RAR or RXR specifically precipitated the intron DR-2 sequence, demonstrating that the element is occupied by the heterodimers in cells. Taken together, the observations establish that caspase 9 is a direct target for RAR signaling, and that the RARE responsible for this response is likely to be a DR-2 element located in the second intron of the gene.

RA affect on cell cycle in MCF-7 cells

Inhibition of MCF-7 cell growth by RA appears to be a multifaceted process involving the induction of both apoptosis and cell cycle arrest (*11-13*). The present study was undertaken in order to obtain insights into the initial cell cycle response, identify direct RAR target genes that are involved in this response, and examine the contribution of CRABP-II to the process. MCF-7 mammary carcinoma cells were treated with RA for 24 or 72 hr, and the cell cycle distribution examined using a 5-bromo-2'-deoxyuridine (BrdU) incorporation assay. Cells were also stained with propidium iodide, and the fraction of cell populations in different cell cycle phases was determined by flow cytometry. The data (Fig. 3) showed that RA treatment resulted in a marked increase in the cell population in the G_1 phase leading to an overall two fold increase at 72 hr. The increase was accompanied by a corresponding decrease in residency in S and in G_2/M phases, demonstrating that RA inhibits the G_1 to S transition, thereby arresting cells in G_1. In addition to a marked effect on cell cycling, RA also triggered DNA fragmentation, reflected by an increase of the fraction of cells in the sub-G_1 population.

Figure 3. MCF-7 cells undergo arrest in G1 in response to RA

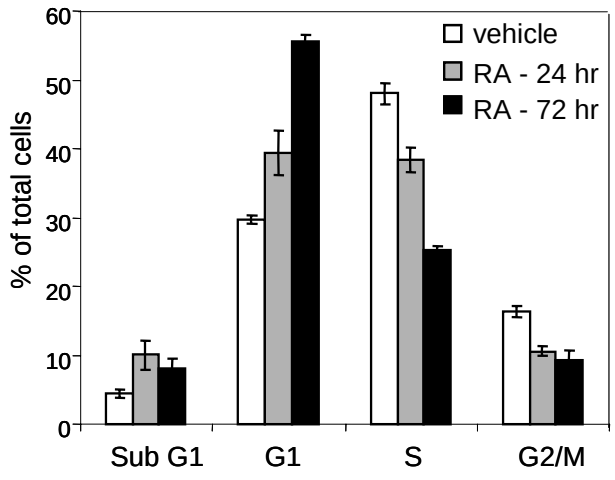
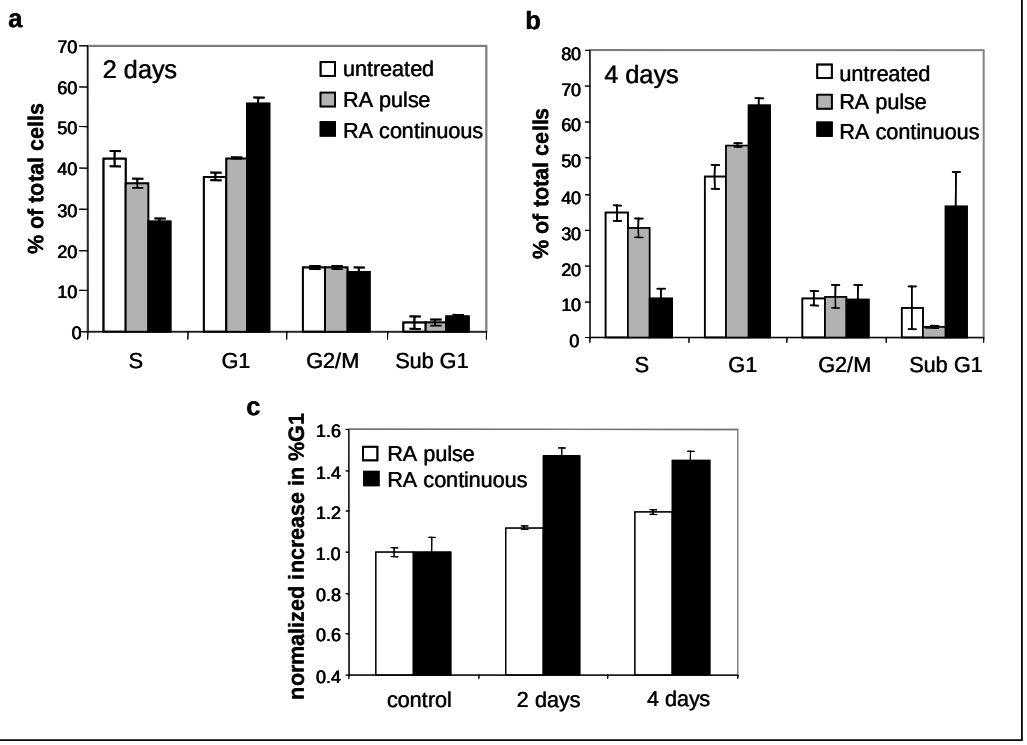
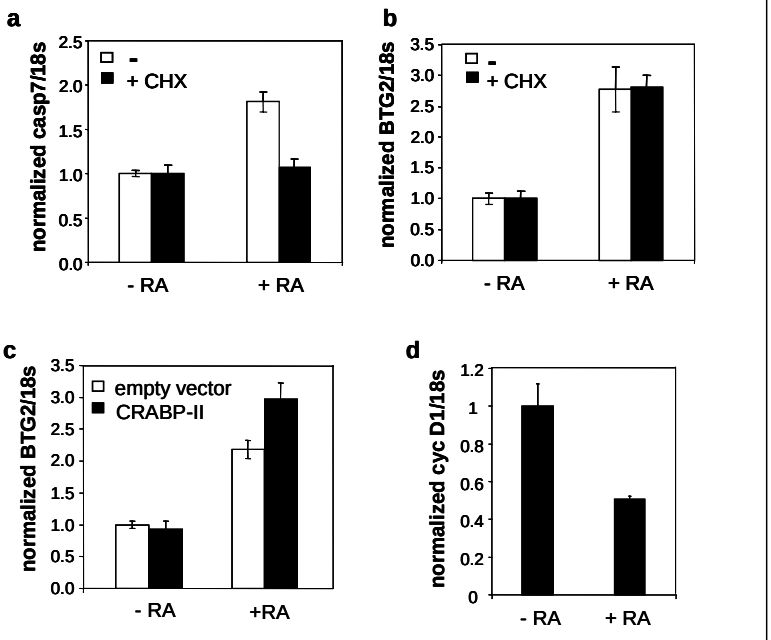


Figure 4. A RA 'pulse' is sufficient to trigger cell cycle arrest in MCF-7 cells



To examine whether a short-term treatment with RA is sufficient for triggering a subsequent cell cycle arrest, or whether the response requires the continuous presence of the ligand, MCF-7 cells were treated with RA, the ligand removed from the medium, and cellular responses were monitored. MCF-7 cells were either pulsed with RA for 3-5 hr. or continuously exposed to the hormone for the entire duration of the experiments, and cell cycle distribution was analyzed at 2 or 4 days following treatment initiation (Fig. 4). In cells pulsed with RA, a significant G_1 cell cycle arrest, reflected by an increase in the fraction of cells in G_1 and a corresponding decrease in cells in the S phase, was evident after 2 days (Fig. 4a), and became more pronounced after 4 days (Fig. 4b). Hence, activation of initial targets of RA-signaling appears to be sufficient for triggering a subsequent cell cycle arrest program in MCF-7 cells. Continuous treatment with RA resulted in a somewhat more enhanced arrest response as well as in induction of apoptosis, which became evident after day 4. In contrast, a short-term exposure to RA was not sufficient to elicit an apoptotic response within the duration of these experiments. It is worth noting however that the cell cycle response to the pulsed treatment appeared to be merely delayed rather than dampened (Fig. 4c). It is thus possible that the ultimate apoptosis response is delayed and would have become evident at later times.

Figure 5. Btg2 is a direct target for RA signalling, and its upregulation is accompanied by a decrease in cyclin D1 expression



To identify genes that may mediate the RA-induced cell cycle arrest, we searched our Affymetrix expression array experiment described above. Several genes that are known to be involved in cell cycle regulation were identified. Among these, B-cell translocation gene, member 2 (Btg2), displayed a greater than 2-fold increase in expression in response to RA. Btg2, (also known as *Tis21*, *Pc3*, or *APRO1*) belongs to the antiproliferative (APRO) family, and is known to function in regulating the G1 to S progression (*14*). Correspondingly, decreased Btg2 expression has been linked to cancerous states (*15, 16*). BTG2 expression in response to RA was verified by quantitative real-time PCR.

Regulation of expression of RA-responsive genes may be exerted directly, i.e. mediated by an RAR response element (RARE). Alternatively, responses may

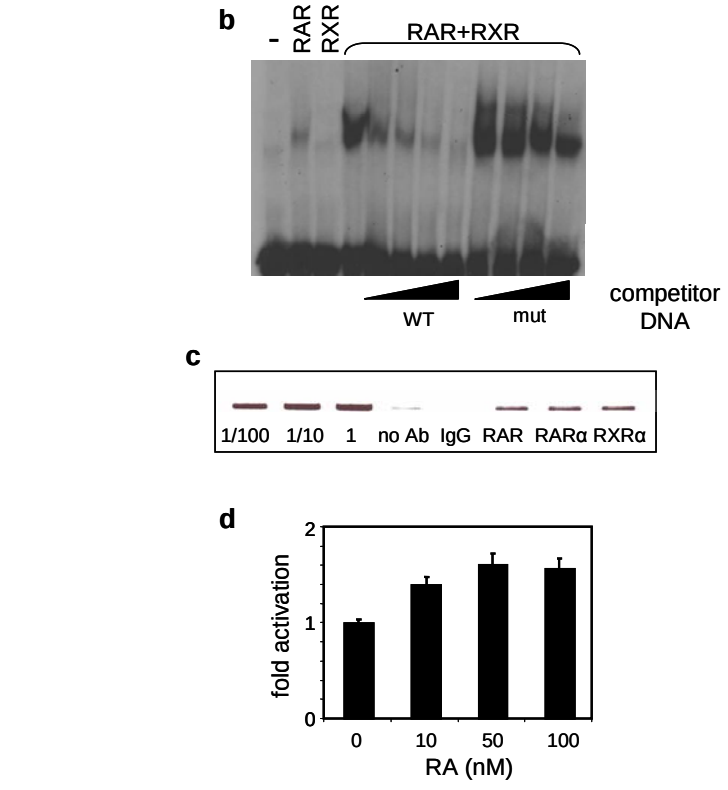
reflect secondary events involving RAR control of immediate target genes, which, in turn, are involved in downstream events leading to the observed modulation. Hence, an important question that arises is whether Btg2 comprises a direct target for RAR. Similarly to the above experiment on caspase 9 expression, to determine whether Btg2 is a direct target for RAR, the effect of cycloheximide treatment on Btg2 level was examined. Cells were pre-treated with cycloheximide and then treated with RA (50 nM) for 4 hr., and Btg2 mRNA expression levels determined. As a control, we examined the effect of cycloheximide on the ability of RA to up-regulate the expression of *caspase-7*, an indirect target for RAR (*8*). The analysis showed that while upregulation of the indirect target *caspase-7* was abolished upon cycloheximide treatment (Fig. 5b), inhibition of protein synthesis did not hinder the ability of RA to enhance the expression of *Btg2* (Fig. 5a). Hence, the observations demonstrate that *Btg2* is a direct target for RAR.

We previously showed that the RA-binding protein CRABP-II directly delivers RA from the cytosol to RAR in the nucleus. Consequently, CRABP-II enhances the transcriptional activity of the receptor both in the context of a reporter gene driven by an RARE (*7*), and in the context of the endogenous direct RAR target gene *caspase-9* (*8*). As the data demonstrated that Btg2 is a direct target for RAR signalling, it was of interest to examine whether upregulation of its expression in response for RA is augmented by CRABP-II. To this end, MCF-7 cells were transfected with an expression vector encoding CRABP-II, and treated with vehicle or RA for 4 hr. The levels of Btg2 mRNA were then examined by Q-PCR (Fig. 5c). The data showed that ectopic over-expression of CRABP-II significantly enhanced the RA-induced upregulation of the expression of the gene. Notably, CRABP-II over-expression alone did not increase the expression of Btg2, indicating that CRABP II does not regulate Btg2 expression independently of RA.

It was previously reported that inhibition of the G1 to S transition by Btg2 involves down-regulation of the expression of cyclin D1 (*14, 17*). The observations that RA up-regulates the expression of Btg2 suggest that RA-induced cell cycle arrest in MCF-7 cells may be mediated, at least in part, by down-regulation of cyclin D1. In support of this notion, we found that a 24 hr. treatment with RA resulted in a 2-fold decrease in cyclin D1 expression in MCF-7 cells (Fig. 5d).

Consensus RAREs consist of two direct hexameric repeats of the sequence PuG(G/T)TCA spaced by either 2 or 5 bp (DR2 and DR5, respectively). To identify the response element that might mediate the RA-responsiveness of Btg2, a 4 Kbp stretch upstream of the transcription start site of the gene was screened for potential RAREs (TransFac, www.gene-regulation.com). Three DR2 half-sites were found to be present 3,250 bp upstream of the start site (Fig. 6a). This element, along with flanking sequences on both sides to a total of 95 bp was generated, labeled with ^{32}P, and its ability to bind RXR-RAR heterodimers examined by electrophoretic mobility-shift assays (EMSA). RAR and RXR lacking their N-terminal A/B domain (RARαΔAB and RXRαΔAB) were bacterially expressed and purified, and the proteins incubated with the 95 bp oligonucleotide containing the Btg2 RARE. Protein-DNA complexes were

Figure 6. A functional RARE in the *Btg2* promoter



resolved by non-denaturing PAGE and visualized by autoradiography (Fig. 6b). Neither RAR nor RXR alone efficiently shifted the mobility of oligonucleotide. Addition of both receptors resulted in the appearance of a shifted band, reflecting binding of the heterodimer to the element. The observations that a cold probe containing the putative element effectively competed for binding to the RXR-RAR heterodimer while the same 95 bp fragment mutated at the RARE did not, further demonstrated the specificity of the interactions.

To determine if the RAR/RXR heterodimer binds to the Btg2 RARE in cells, chromatin immunoprecipitation assays (ChIP) were carried out. Proteins were cross-linked to chromatin in MCF-7 cells, sonicated, and immunoprecipitated using antibodies for RAR or RXR. A 200 bp region flanking the Btg2 RARE was amplified by PCR to detect receptor binding. The data (Fig. 6c) showed that antibodies against either RAR or RXR precipitated the putative Btg2 RARE, indicating that the element is occupied by the RXR-RAR heterodimer in cells.

To examine the functionality of the putative RARE, the 95 bp oligonucleotide containing the element was cloned upstream of a luciferase reporter, the reporter transfected into MCF-7 cells, and transactivation assays were carried out. The data (Fig. 6d) demonstrated a dose-responsive activation of reporter expression by RA, indicating that the element indeed comprises a functional RARE.

Previous reports (*8, 12, 13*) and the data presented here indicate that RA-induced growth inhibition of mammary carcinoma MCF-7 cells comprises a two-phase process, entailing an early cell cycle arrest followed by induction of apoptosis. We have identified some of the mechanisms by which RA induces both cell cycle arrest and apoptosis in MCF-7 cells.

Development of an optical assay to measure RA from biological samples

Retinoic acid (RA) and various fatty acid derivatives activate the nuclear receptors RARs and PPARs, respectively, and thus modulate transcription of numerous genes, play key roles in multiple biological processes, and are utilized in therapy of a number of diseases. However, therapy with these agents is often confounded by toxicity, raising the need for their ready quantitation in biological samples. To develop an optical method for quantitating RA in biological samples, we considered CRABP-I and CRABP-II. These proteins are highly selective towards RA, they bind their ligand with equilibrium dissociation constants (Kd) in the sub-nM range (*6*), and they are readily obtained by bacterial expression. Association of RA with RA-binding proteins is often monitored by following the intrinsic fluorescence of the proteins emanating from tryptophan and tyrosine residues (*6, 18*). As the emission spectra of proteins extensively overlaps with the absorption spectrum of RA, the intrinsic fluorescence of RA-binding proteins is quenched upon their ligation. Titration with RA thus results in a decrease in protein fluorescence until the protein is saturated and a plateau is reached (Fig. 7). Resulting titration curves can then be analyzed to yield the Kd of the ligand-protein interactions. However, while the intrinsic fluorescence of a protein can be readily used to monitor ligand binding in highly purified systems, utilizing this approach to measure RA concentrations in biological samples such as extracts from cells, plasma, and tissues is complicated by the presence of other fluorescent compounds in the extracts. In addition, the quantum yield of the intrinsic fluorescence of proteins is quite low, limiting the sensitivity of the method.

Figure 7. Fluorescence titrations of CRABP-I and CRABP-II

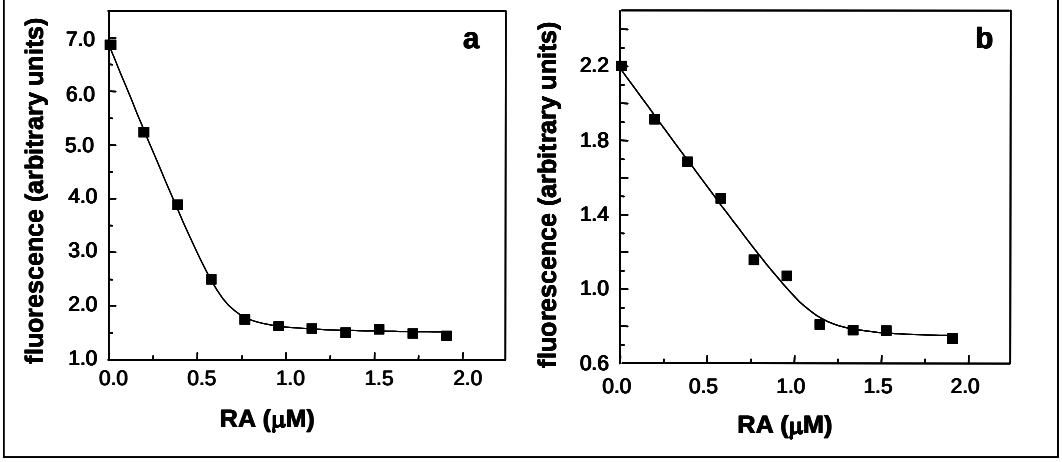
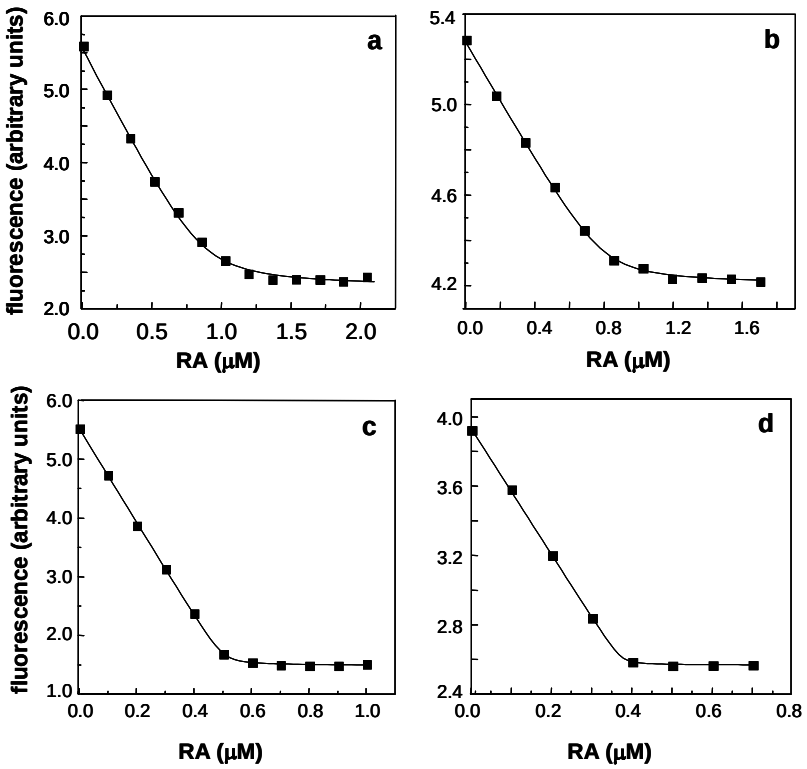


Figure 8. X-ray crystal structures of apo- and holo-CRABP-II

Figure 9. Fluorescein-labeled CRABP-II-L28C and CRABP-I-L-28C can serve as sensors for RA



These limitations may be overcome if the protein can be covalently labeled with a fluorescent probe whose fluorescence properties change upon ligand-binding by the protein. Utilization of a probe with a high fluorescence quantum yield and with an emission maximum at long wavelengths will allow for higher sensitivity, and for measurements at wavelengths that are far from emission spectra of many biological fluorophores, minimizing the optical background. In addition, using such a responsive probe may enable monitoring binding of retinoids that, unlike RA, lack useful optical properties, thereby expanding the utility of the method. With these considerations, we attempted to label CRABP-I and CRABP-II using a variety of protein-modifying fluorescent reagents, including reagents that attack amines, carboxylic acids, and thiols. However, none of these led to efficient labeling of either of the proteins, suggesting that they lack reactive residues that are accessible to covalent modification. We thus set out to engineer CRABP mutants in which a readily-modifiable residue is introduced into a region of the proteins that is expected to undergo a conformational change upon ligand-binding. To identify such a region, we examined the previously reported x-ray crystal structures of apo-CRABP-II ((*19*), PDB entry 1XCA) and of the RA-bound protein ((*20*), PDB entry 1CBS). Comparison of the two structures (Fig. 8) revealed that, while the overall structures of the apo- and the holo-protein are strikingly similar, subtle differences can be observed within the helix-loop-helix region of the protein. As previously noted for other iLBPs (*21*, *22*), this 'portal' region (boxed in Fig. 8), which forms a lid over the entry to the binding site of the protein, shifts slightly upon ligand binding. In particular, we targeted residue L28 whose side chain is placed at a different configuration in apo- *vs*. holo-CRABP-II (Fig. 8). As leucines are not readily modified, we exchanged this residue with a cysteine to generate a CRABP-II-L28C mutant.

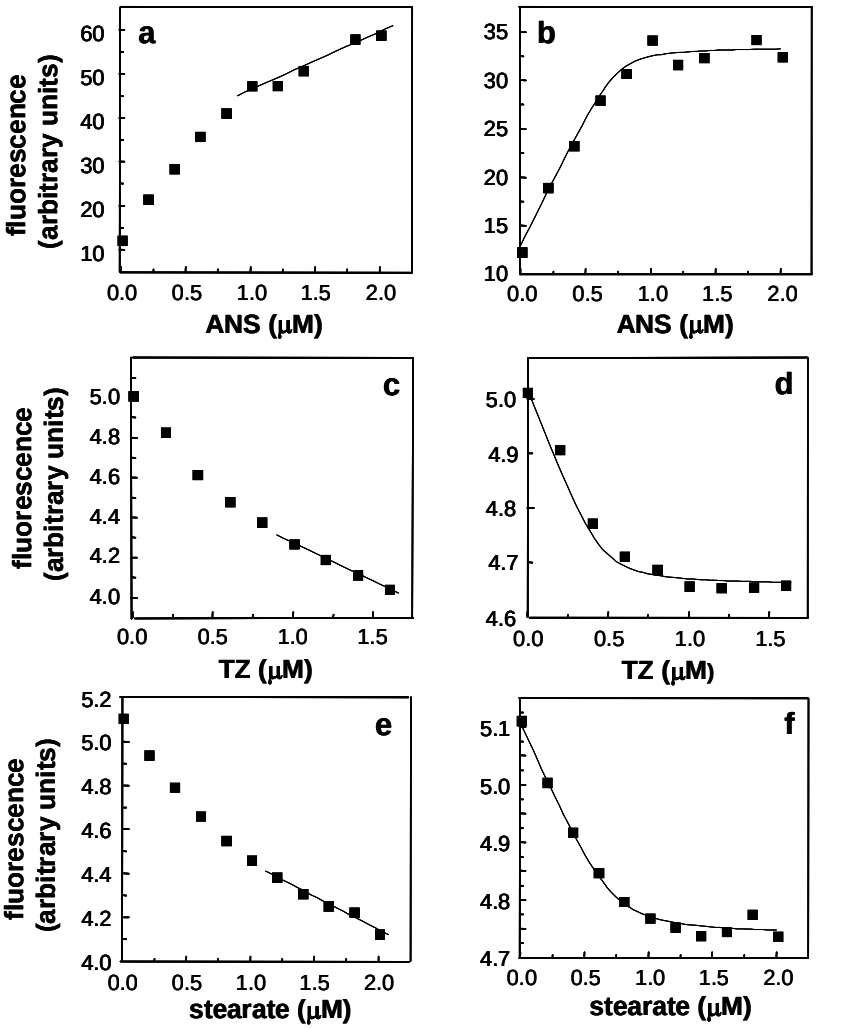
CRABP-II and its L28C mutant, fused with a hexahistidine tag, were expressed in *E. coli*, and purified using metal chelating affinity chromatography. The association of purified WT and mutant proteins with RA was then examined by conventional fluorescence titrations in which the decrease in the intrinsic fluorescence of the proteins is followed (Fig. 7b and Fig. 9a, respectively). Analyses of resulting titration curves (*18*) yielded Kds of 10±3.6 nM, and 44±5.4 nM (mean±SEM, n=4) for the WT protein and its mutant counterpart, respectively. It should be noted that, because of sensitivity limitations, these titrations are carried out at protein concentrations that are higher than the dissociation constant and thus, that the derived Kd reflects an upper limit for the actual value. Nevertheless, the data show that the mutant retaines tight association of CRABP-II with RA. CRABP-II-L28C was then covalently labeled with the environmentally-sensitive fluorescent probe

fluorescein using the thiol-reactive 5-(bromomethyl)fluoroscein (BMF) reagent. The resulting labeled mutant was titrated with RA, and the fluorescence of the protein-bound probe was monitored. The data (Fig 9b.) showed that fluorescence of the probe decreased upon addition of RA to attain a plateau, reflecting protein saturation. Analyses of titration curves obtained by monitoring probe fluorescence (λex – 492 nm; λem – 520 nm) yielded a Kd of 18±0.5 nM (mean±SEM, n=3), essentially identical to values obtained by following the intrinsic fluorescence of the protein. Hence, changes in fluorescence the CRABP-II-bound probe reliably report on ligand-binding, establishing that it may serve as a sensitive 'read-out' for RA.

A corresponding mutation was made in CRABP-I. Histidine-tagged CRABP-I-L28C was purified from over-expressing *E. coli*, and labeled with fluorescein. Fluorescence titrations of WT-CRABP-I (Fig. 7a) and of the labeled mutant (Fig. 9c) carried out by monitoring the intrinsic fluorescence of the protein demonstrated that proteins associated with RA with Kds of 7 nM (mean of two measurements) and 2.5±0.7 nM (mean±SEM, n=3), respectively. Monitoring changes in the fluorescence of the CRABP-I-attached fluorescein upon titration with RA further demonstrated that the probe readily reported on ligand-binding by the protein (Fig. 9d). Kd obtained from titrations followed by monitoring probe fluorescence had a value of 4.7±2.6 nM (mean±SEM, n=4). Hence, fluorescently-labeled CRABP-I as well as CRABP-II may be used to detect RA.

Members of the iLBP family share a highly conserved 3-dimensional fold, and examination of crystal structures of various iLBPs suggest that ligand-induced structural shifts within their portal regions may be a common feature of these proteins (*22*). These considerations raise the possibility that attaching a fluorescent probe at this region of iLBPs other than CRABPs may allow for development of optical assays for studying ligands that associate with them. Indeed, it was reported that the fluorescence of a tryptophan residue inserted at position 28 of liver FABP changes in respond to ligand-binding (*23*), and that fatty acid binding by intestinal-FABP labeled with the fluorescence probe acrylodan at Lys27 induces a shift in the spectrum of the probe (*24*). However, while FABPs associate with a remarkably broad spectrum of ligands, at least two of these proteins are 'activated', i.e. induced to undergo nuclear localization, only by specific compounds (*25*). A functionally meaningful assay will thus require an optical method that not only reports on ligand-binding, but that allows for discriminating between 'activating' *vs*. 'non-activating' ligands.

Figure 10. Fluorescein-labeled A-FABP-T29C can serve as an non-discriminatory sensor for A-FABP ligands



We thus set out to generate an optical assay for ligands that associate with A-FABP, an iLBP that binds a variety of lipophilic compounds, but whose nuclear localization is specifically activated by ligands for the nuclear receptor PPARγ, such as the synthetic drug troglitazone (*25*). Thr29, the A-FABP residue that corresponds to CRABP-II-L28 was exchanged for a cysteine to generate an A-FABP-T29C

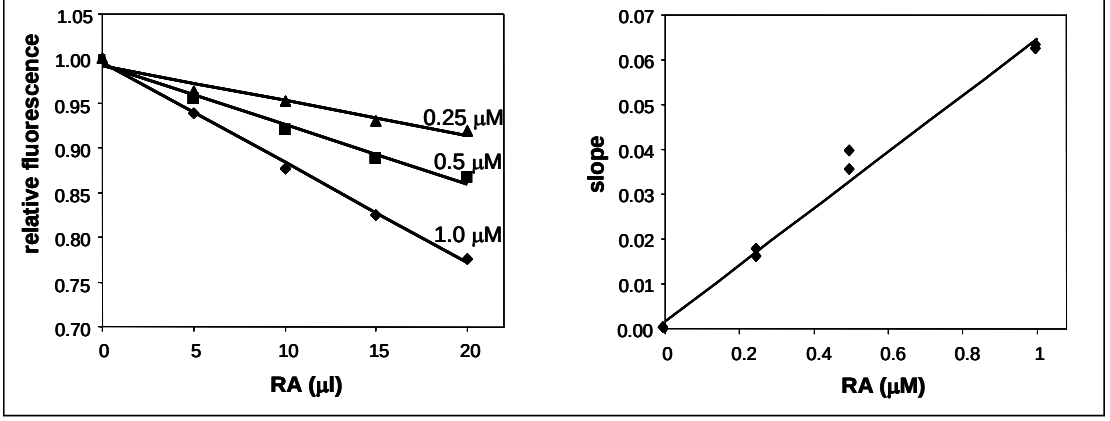
mutant. The mutant was purified from over-expressing *E. coli*, and labeled with fluorescein using procedures similarly to those used for labeling the CRABPs. To examine the ligand-binding characteristics of the labeled A-FABP-T29C, Kd for its association with the fluorescent fatty acid probe anilinonaphtalene-8-sulphonic acid (ANS) was measured. ANS readily associates with various FABPs and its fluorescence is highly enhanced upon binding. Hence, it has been widely used to study ligand-binding by FABPs (*26, 27*). Titration of fluorescein-labelled-A-FABP-T29C with ANS showed a saturable increase in ANS fluorescence (Fig. 10a). Continuing addition of the probe following saturation resulted in an additional linear increase reflecting the fluorescence of unbound probe added to mixtures (*28*). Titration curves were corrected for the linear fluorescence increase, and corrected curves (Fig. 10b) were analyzed to derive the Kd for association of ANS with A-FABP. Kd for the association was found to be 56 nM (mean of 2 measurements), well within the 20-70 nM range previously reported to characterize the association of A-FABP with other ligands (*25*).

To examine the response of fluorescein-labeled A-FABP to binding of non-fluorescent ligands, fluorescence titrations were carried out with two compounds: troglitazone, which activates the nuclear localization of A-FABP, and stearate, a long-chain fatty acid that binds to A-FABP but is a 'non-activating' ligand for this protein. The labeled protein was titrated with either ligand, and the fluorescence of the protein-bound probe monitored (Fig. 10c and 10e). Titrations with either ligand resulted in a saturable decrease in the fluorescence of the labeled protein followed by a linear decrease. Curves were corrected for the non-specific linear decrease, and corrected curves (Fig. 10d and 10f) analyzed to yield Kds of 26 nM and 38 nM for association of A-FABP with troglitazone and stearate, respectively. Hence, the fluorescence of the A-FABP-bound probe efficiently reports on ligand-binding by the protein but does not discriminate between 'activating' and 'non-activating' A-FABP ligands.

As an example for utilization of the newly developed assay for quantitating of RA in biological samples, we examined the synthesis of RA in cultured mammalian cells. Two cell lines were used: COS-7 cells, and the mammary carcinoma MCF-7 cells which were reported to be impaired in RA synthesis (*29*), and used here as a negative control. To initiate RA synthesis, cells were treated with retinal, the metabolic precursor for RA. Following incubation for different time periods, media containing RETINAL was removed, cells were washed, collected by centrifugation, and ligands extracted into ethanol. Ethanol extracts were used in subsequent measurements.

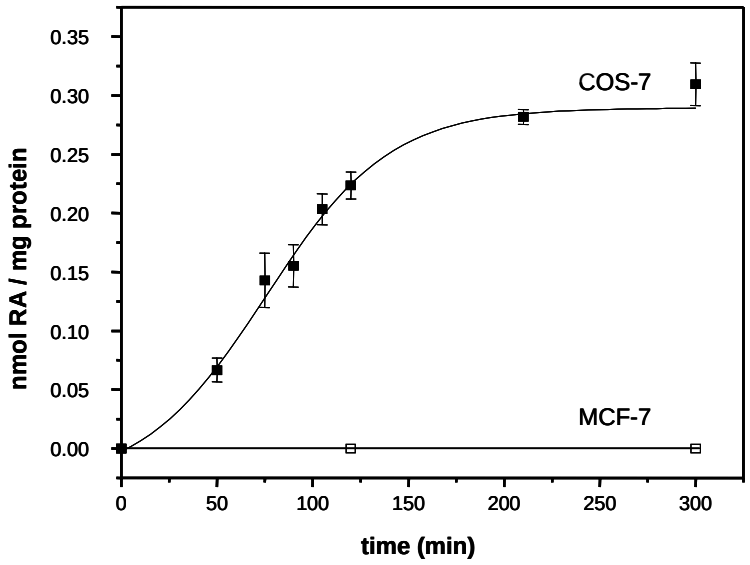
To obtain a calibration curve, RA standard solutions were generated by extracting untreated cells with ethanol containing known RA concentrations. Fluorescein-labeled CRABP-I-L28C (0.05 μM) was then titrated with these standards. As expected, the titrations displayed steeper initial slopes as the RA concentration in the standard solution was increased (Fig. 11a). A calibration curve was then constructed by plotting the absolute values of the initial slopes as a function of the RA concentration in standard solutions (Fig. 11b). The linearity of the calibration curve demonstrates the reliability of the assay up to 1 μM RA. Titration of CRABP-I-L28C with cell extracts that did not contain exogenously added RA did not display a detectable slope, demonstrating very low levels of endogenous RA in these cells. To monitor RA synthesis, titrations were carried out using ethanol extracts derived from retinal-treated cells. Initial slopes were obtained for each titration, and the concentration of RA in each extract calculated based on the calibration curve, and normalized to the amount of protein in extracted cells. Time

Figure 11. Construction of a calibration curve for quantitating RA in cells



courses for RA synthesis in RETINAL-treated COS-7 and in MCF-7 cells (Fig. 12) demonstrated that, COS-7 cells readily synthesize RA, reaching a steady state concentration of about 0.3 mole RA/mg protein within 140 min. of treatment with 2 μM retinal. In contrast, under the same conditions, the level of RA in MCF-7 remained undetectable.

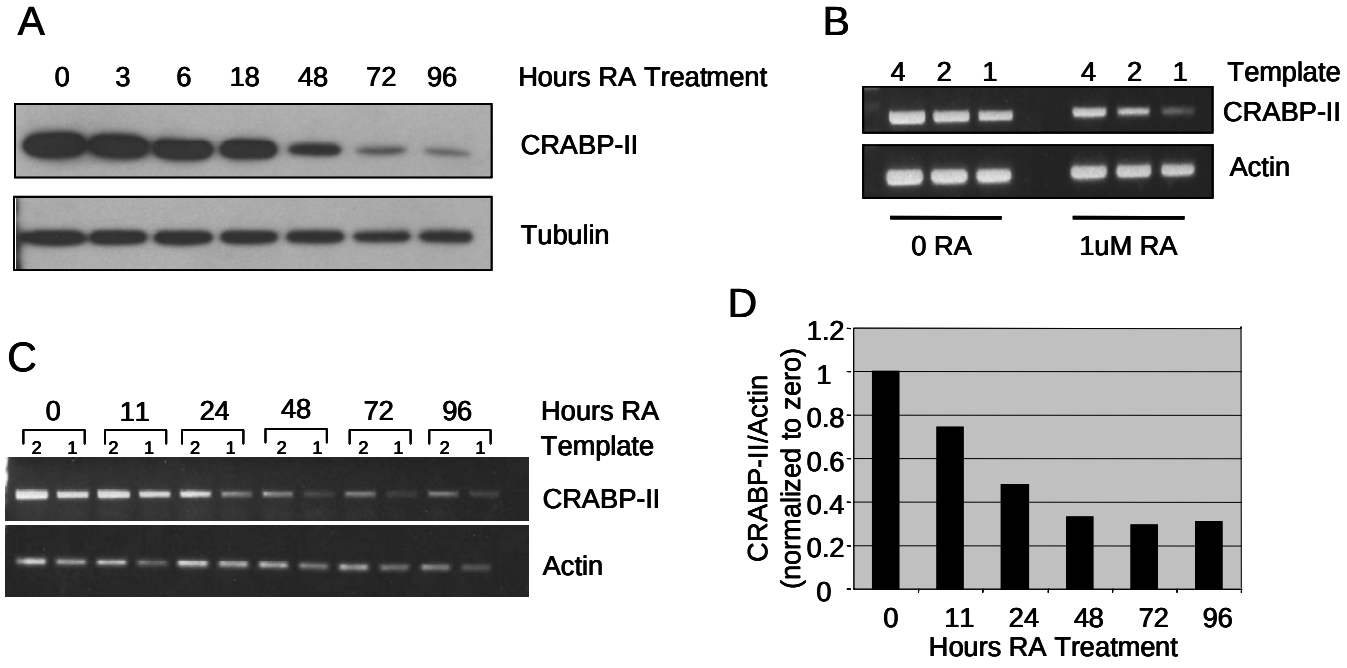
Figure 12. RA synthesis in MCF-7 and COS-7 cells



Understand the underlying basis for RA resistance in mammary carcinoma cells

A frequent complication in utilizing RA for cancer therapy is the development of RA-resistance in tumors. Understanding the mechanisms that underlie RA-resistance in cancer cells are thus of significant clinical importance. We have observed that mammary carcinoma cells that over-express CRABP-II become more sensitive to RA-induced growth inhibition. Conversely, we find that cells that do not express CRABP-II are resistant to this anti-proliferative effect. Therefore, we examined the effect of RA on the expression of CRABP-II. The data reveal that RA treatment resulted in a decrease in CRABP-II protein in MCF-7 cells (Figure 13A). This effect may originate from two possible responses: (1) RA treatment may lead to a degradation of CRABP-II protein, or (2) RA treatment may result in a decrease in mRNA levels. To determine whether this loss of protein is due to a decrease in CRABP-II mRNA level, semi-quantitative PCR was performed on extracts from cells treated with RA. Figure 13B-D shows that the CRABP-II RNA level in MCF-7 cells is down regulated in response to RA treatment. RA also causes CRABP-II protein and mRNA levels to decrease in other cells including the human keratinocyte cell line

Figure 13. RA treatment causes reduced expression of CRABP-II



HaCat (Figure 14). This down-regulation of CRABP-II in response to RA suggests a novel mechanism of negative feedback regulation of RA signaling. The decrease in CRABP-II mRNA levels could arise by two different mechanisms: (1) RA could affect the rate of transcription of the CRABP-II gene (2) RA could modulate the stability of the CRABP-II mRNA. It has been previously shown that RAR itself is degraded in response to RA treatment via the ubiquitin-proteosome pathway. CRABP-II is regarded as a RA-induced gene controlled by an RARE in its promoter region. Therefore, we hypothesized that RA could decrease CRABP-II mRNA levels by causing the degradation of the transcription factor (RAR) that is needed for its expression. To test this hypothesis, we transfected exogenous RAR into MCF-7 cells and monitored the CRABP-II protein and mRNA levels to see if replenishing RAR levels would rescue CRABP-II expression. Even in cells that over-express RAR, CRABP-II protein and mRNA levels continued to decrease in response to RA (Figure 15). Therefore, replenishing RAR did not rescue CRABP-II expression.

Figure 14. RA causes down-regulation of CRABP-II in HaCat cells

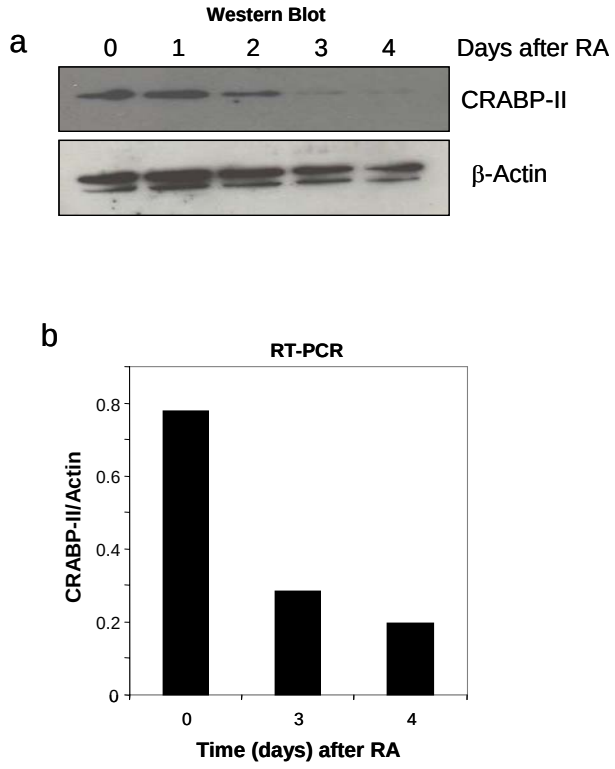
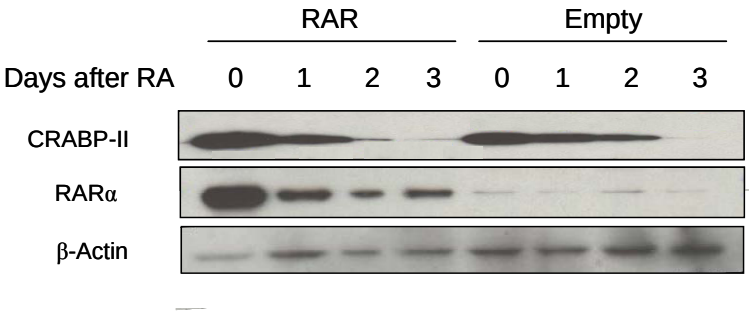
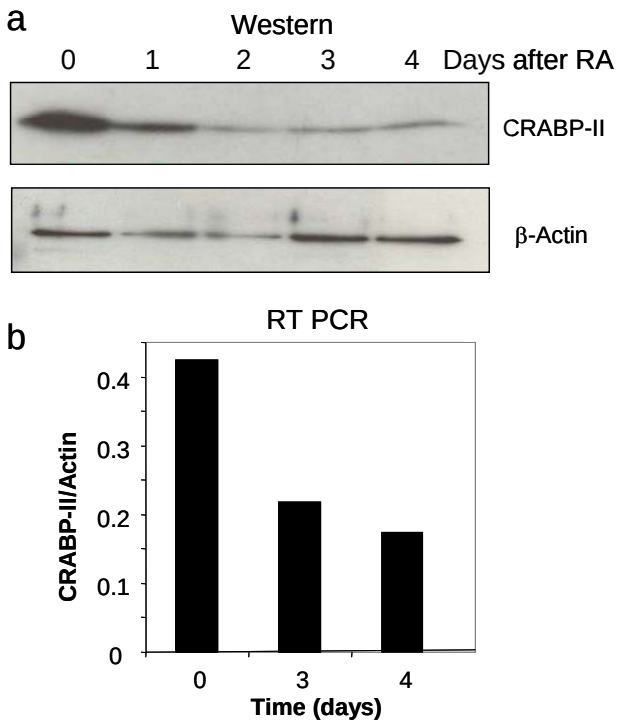


Figure 15. Exogenous RAR does not rescue CRABP-II expression



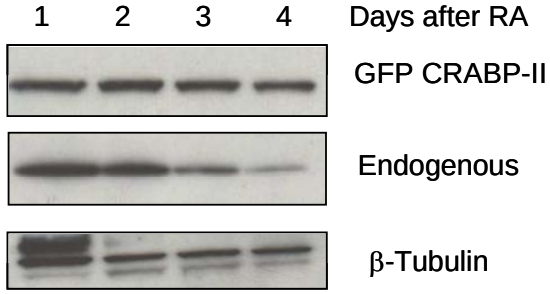
To test directly if CRABP-II mRNA levels decrease because of a decline in the transcriptional rate of the endogenous gene, we utilized a cell line that does not express endogenous CRABP-II, but that is stably transfected with the CRABP-II cDNA. In this cell line (SC115.C1), the expression of CRABP-II is not under the control of its native promoter. If RA affects the rate of transcription of the CRABP-II gene, we would expect the transcript level in this cell line to be unresponsive to RA treatment. The date showed that CRABP-II protein and mRNA levels decrease in response to RA in the SC115.C1 cell line (Figure 16). This result suggests that CRABP-II mRNA levels are not affected by RA at the transcriptional level and points to mRNA stability as the target of RA actions on the CRABP-II transcript.

Figure 16. CRABP-II down-regulation by RA in SC115.C1 cells



Message RNA stabilities are often regulated by RNA-binding proteins, many of which bind to the 3' untranslated region (UTR). One such protein, nucleolin, has been shown to stabilize the BCL-2 transcript by binding to an AU-rich region in the transcripts 3'-UTR(*30*). It has also been shown that upon treatment with RA, nucleolin levels decrease resulting in destabilization of the BCL-2 transcript and reduction of its expression level (*31*). The CRABP-II transcript has a similar AU-rich region in its 3' UTR. To test whether the UTRs of the CRABP-II transcript are important for its stability, we transfected MCF-7 cells with a GFP-tagged CRABP-II construct that does not contain the gene's untranslated regions. Upon treatment with RA, the endogenous levels of CRABP-II decreased as have been observed before; however, the GFP-CRABP-II construct was unresponsive to RA treatment (Figure 17). These observations indicate that the UTRs of CRABP-II could be responsible for the stability of the transcript. RA-induced down-regulation of CRABP-II expression may thus comprise an important feature through which carcinoma cells are rendered RA-resistant. On going studies aim to test the effects of CRABP-II UTRs in its mRNA stability. RA-induced down-regulation of CRABP-II expression may comprise an important feature through which carcinoma cells are rendered RA-resistant.

Figure 17. GFP-CRABP-II is not down-regulated by RA



Key Research Accomplishments

- Caspase 7 activation is induced upon RA-induced apoptosis in MCF-7 cells.
- Caspase 9 is a direct target for RA signaling and CRABP-II enhances its expression. This is the first demonstration of CRABP-II facilitating RAR-mediated transcription of an endogenous gene.
- Caspase 9 contains a functional RARE in its second intron.
- Over-expression of CRABP-II in MCF-7 cells enhances the caspase 7-mediated apoptotic response to RA.
- RA induces G1 cell cycle arrest after 1-3 days of treatment.
- RA-induced cell cycle arrest involves induction of BTG2.
- BTG2 is a novel direct target for RA and its expression is enhanced upon over-expression of CRABP-II.
- Development of a novel optical assay for detection of RA from biological samples.
- RA induces the down-regulation of CRABP-II expression in a variety of cells.
- RA-mediated down-regulation of CRABP-II is not exerted through effects on the transcription of the gene.
- RA-mediated down-regulation of CRABP-II may be due to decreasing its mRNA stability.
- RA-resistance of carcinoma cells may stem from RA-induced down-regulation of CRABP-II.

Reportable Outcomes:

- **Donato LJ** and Noy N. Quantitation of Retinoic Acid in Biological Samples Using a Fluorescence Based Assay, *in preparation*
- **Donato LJ**, Suh JH, and Noy N. Suppression of mammary carcinoma growth by retinoic acid: cell cycle control genes are targets for RAR and CRABP-II signaling, Cancer Res., *in review*
- **Donato, LJ** and Noy, N. Suppression of mammary carcinoma growth by retinoic acid: pro-apoptotic genes are targets for RAR and CRABP-II signaling in MCF-7 cells, Cancer Res. 2005 Sep 15;65(18):8193-9

*note- Leslie Willmert changed her name to Leslie Donato (marriage)

Conclusions:

RA is currently used or is in clinical trials for therapy of a variety of cancers, however, at pharmacological doses it is often toxic and tumors often develop resistance to the treatment. Our lab is investigating approaches that will allow for sensitization of cancer cells to RA chemotherapy in order to increase the therapeutic efficacy of this compound. We have found that the RA binding protein CRABP-II functions to inhibit mammary carcinoma cell proliferation in culture as well as tumor progression *in vivo*. Therefore, CRABP-II may be a novel target for therapeutic and preventive strategies for treatment of breast cancer. The mechanisms by which this binding protein acts to modulate RA signaling and enhance the anti-proliferative activities of this compound in breast cancer are being studied. CRABP-II enhances RA-induced apoptosis in MCF-7 cell via caspase-mediated events. One of these caspase, caspase 9, was shown to be a novel direct target for RA signaling. CRABP-II also enhances RA-induced G1 cell cycle arrest in MCF-7 cells via an increase in BTG2 expression and down-regulation of cyclinD1 expression. BTG2 was shown to be a novel direct target for RA. During this funding period, we have developed a novel assay for detection of RA from biological samples. Such a method can be used in monitoring RA synthesis or degradation in experimental systems or possibly to monitor serum levels in patients treated with RA. Additional studies carried out during this funding period have begun to elucidate the mechanism by which RA down-regulates CRABP-II expression in carcinoma cells. This effect is not due to decreased transcriptional activation of the gene, but rather, RA affects the stability of the CRABP-II mRNA. This activity may be an important factor in the development of RA-resistance in carcinomas.

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Suppression of Mammary Carcinoma Growth by Retinoic Acid: Proapoptotic Genes Are Targets for Retinoic Acid Receptor and Cellular Retinoic Acid–Binding Protein II Signaling

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Abstract

Retinoic acid (RA) displays pronounced anticarcinogenic activities in several types of cancer. Whereas the mechanisms that underlie this activity remain incompletely understood, tumor suppression by RA is believed to emanate primarily from its ability to regulate transcription of multiple target genes. Here, we investigated molecular events through which RA inhibits the growth of MCF-7 mammary carcinoma cells, focusing on the involvement of the two proteins that mediate transcriptional activation by RA, the nuclear hormone receptor retinoic acid receptor (RAR) and the cellular retinoic acid-binding protein (CRABP) II, in this process. RA treatment of MCF-7 cells did not affect cell cycle distribution but triggered pronounced apoptosis. Accordingly, expression array analyses revealed that RA induces the expression of several proapoptotic genes, including *caspase 7* and *caspase 9*. Whereas *caspase 7* is an indirect responder to RA signaling, *caspase 9* is a novel direct target for RAR, and it harbors a functional retinoic acid response element in its second intron. In agreement with the known role of CRABP-II in enhancing the transcriptional activity of RAR, the binding protein augmented RA-induced up-regulation of *caspase 9*, cooperated with RA in activating both caspase 7 and 9, and amplified the ability of RA to trigger apoptosis. Surprisingly, the data indicate that CRABP-II also displays proapoptotic activities on its own. Specifically, overexpression of CRABP-II, in the absence of RA, up-regulated the expression of Apaf1 and triggered caspase 7 and caspase 9 cleavage. These observations suggest that, in addition to its known role in direct delivery of RA to RAR, CRABP-II may have an additional, RA-independent, function. (Cancer Res 2005; 65(18): 8193-9)

Introduction

Retinoic acid (RA), the active metabolite of vitamin A, regulates multiple biological processes, including cell proliferation, differentiation, and death, and thus plays critical roles in embryonic development and in growth and remodeling of adult tissues. Natural and synthetic RA derivatives, collectively known as retinoids, are also potent inhibitors of cancer cell growth and have been shown to be efficacious in therapy and prevention of various types of cancer (1, 2). The pathways by which RA inhibits the growth of carcinoma cells seem to vary between cell types. It was thus reported that RA induces differentiation in embryonic tetratocarcinoma F9 cells (3), G_1/G_0 growth arrest and myeloid differentiation in HL-60 cells (4, 5), and postmaturation apoptosis in NB4 acute promyelocytic leukemia cells (6). In mammary carcinoma cell lines, RA was shown to induce growth inhibition by triggering either cell cycle arrest or apoptosis, or both (7–9).

The biological activities of RA are believed to be exerted primarily through the ability of this hormone to regulate gene expression, an activity that is mediated by members of the superfamily of nuclear hormone receptors termed retinoic acid receptors (RARs; ref. 10). Like other type II nuclear receptors, RARs function as heterodimers with the retinoid X receptor (RXR). These heterodimers associate with specific DNA sequences (retinoic acid response elements, RARE) composed of two direct repeats of the consensus sequence PuG(G/T)TCA, separated by either 2 bp (DR-2) or 5 bp (DR-5; refs. 10, 11). RXR-RAR heterodimers thus bind in regulatory regions of their target genes and enhance transcriptional rates on binding of RA (12).

Only limited information is currently available on the identity of immediate RAR target genes that mediate the anticarcinogenic activities of RA. Three such genes were reported to be involved in RA-induced apoptosis and differentiation in NB4 promyelocytic leukemia cells (i.e., ubiquitin-activating enzyme E1–like protein, CCAAT/enhancer binding protein ε, and tumor necrosis factor (TNF)–related apoptosis-inducing ligand; refs. 6, 13, 14). Studies of breast cancer cells suggest that RA-induced apoptosis is associated with down-regulation of Bcl-2 and survivin (15, 16) and with up-regulation of the tumor suppressor gene PDCD4 and of SOX9, a member of the high mobility group box family of transcription factors (17, 18). The mechanisms underlying the RA-responsiveness of these genes and whether any of these comprise direct RAR targets remain to be clarified.

In addition to RAR, two other proteins, termed cellular retinoic acid-binding proteins (CRABP-I and CRABP-II), bind RA with high affinity and specificity (19, 20). CRABPs are small ($\sim$14 kDa) soluble proteins that are members of the family of intracellular lipid binding proteins. Whereas it is generally believed that CRABPs function to solubilize and protect RA in the aqueous space of the cytosol, accumulating evidence suggests that they also play more specific roles in modulating signaling by RA. In regard to the biological functions of CRABP-II, we recently showed that this protein transports RA from the cytosol to the nucleus where it directly associates with RAR. We further showed that the resulting CRABP-II-RAR complex mediates "channeling" of RA to the receptor, thereby facilitating its ligation and enhancing its transcriptional activity (19, 21, 22). Additional observations showed that, as a result of the ability of CRABP-II to augment the transcriptional activity of RAR, overexpression of the protein in mammary carcinoma cells dramatically sensitizes them to RA-induced growth inhibition (21). Correspondingly, CRABP-II was found to inhibit mammary tumor growth both in a xenograft model and in the transgenic breast cancer mouse model mouse mammary tumor virus-*neu* (MMTV-*neu*), in which

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mammary tumors develop spontaneously and progress under immune surveillance (23). Inhibition of breast tumor development in MMTV-*neu* mice treated with CRABP-II was found to stem mainly from increased apoptotic rates (23).

The present study was undertaken to obtain further insights into the mechanisms by which RA induces growth inhibition in the mammary carcinoma MCF-7 cells and the roles of RAR and CRABP-II in this process. We show that treatment of MCF-7 cells with RA has little effect on cell cycle distribution but results in up-regulation of expression of several proapoptotic genes, and in a marked induction of apoptosis. One of the RA-induced proapoptotic genes, *caspase 9*, is shown to be a novel direct target for RAR and to harbor a functional RARE in its second intron. We show that CRABP-II cooperates with RAR in mediating the induction of caspase-9 expression, and that the binding protein enhances the RA-initiated apoptotic response of these cells. Additional observations indicate that, in the absence of RA, CRABP-II up-regulates the mRNA expression level of apoptotic protease activating factor 1 (Apaf1) and triggers cleavage of both caspase 7 and caspase 9. These observations imply that besides its cooperation with RAR in mediating cellular responses to RA, CRABP-II possesses additional, RA-independent, functions.

Materials and Methods

Reagents. Antibodies against caspases were purchased from Cell Signaling (Beverly, MA). Antibodies against mCRABP-II (5CRA3B3) were a gift from Pierre Chambon (IGMCB, Strasbourg, France). Anti-mouse and anti-rabbit immunoglobulin horseradish peroxidase–conjugated antibodies were from Amersham (Arlington Heights, IL).

Cells. MCF-7 cells were purchased from the American Type Culture Collection (Manassas, VA). Cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) or 10% charcoal-treated FBS.

Vectors. The putative RARE in the caspase 9 promoter and the intron DR-2 were separately cloned into the pGL3 luciferase reporter vector (Promega) using the enzyme sites *Kpn*I and *Xho*I.

Proteins. Recombinant histidine-tagged RARα and RXRα lacking the amino terminal A/B domains (RARαΔAB and RXRαΔAB) were expressed in *E. coli* and purified as previously described (24).

Viruses. Adenoviruses were made by the Gene Transfer Vector Core center at the University of Iowa and stored as described (23).

Transactivation assays. MCF-7 cells were transfected with the indicated luciferase reporter plasmid and pCH110 (internal standard) using Fugene (Roche). Following an overnight incubation, cells were treated with RA for 24 hours and lysed. Luciferase expression was assayed using the luciferase assay system (Promega) and corrected for transfection efficiency by the activity of β-galactosidase, which was measured by standard procedures.

Flow cytometry. Cells were seeded in 60 mm plates in DMEM supplemented with 10% charcoal-treated FBS. Cells were transduced with Ad-CRABP-II or Ad-0 virus [multiplicity of infection (MOI) of 500] and grown for 18 hours before addition of RA. RA was replenished every 48 hours. Cells were scraped and collected, washed with cold PBS, lysed in hypotonic buffer (1 mg/L sodium citrate, 0.1% Triton X-100), and propidium iodide (1 mg/mL) was added. Samples were run on a Becton Dickinson FACS Calibur and the results analyzed with CellQuest version 3.3.

Western blotting. Cells were treated as indicated, scraped into the media, and collected. Pellets were washed with cold PBS and lysed in lysis buffer [10 mmol/L potassium phosphate (pH 7.5), 0.5% (w/v) Triton X-100, 10 μg/mL leupeptin, and 10 μg/mL aprotinin]. Protein concentrations in cell lysates were determined by Bradford assay (Bio-Rad) and 30 μg protein loaded in each lane. Proteins were resolved by 12% SDS-PAGE and visualized by Western blots using appropriate antibodies.

Quantitative real-time PCR. RNA was extracted using RNeasy (Qiagen, Valencia, CA). cDNA was generated using Gene Amp RNA PCR (Applied Biosystems, Foster City, CA). Quantitative real-time PCR was done in replicates using TaqMan chemistry and Assays on Demand probes (Applied Biosystems) for caspase 9 (Hs0015426_m1), caspase 7 (Hs00169152_m1), and Apaf1 (Hs00559421_m1). 18s rRNA (4319413E-0312010) was used as a loading control. Analyses were carried out using the relative standard curve method (Applied Biosystems Technical Bulletin no. 2).

Chromatin immunoprecipitation. Nearly confluent MCF-7 cells were treated with 9cRA (1 μmol/L, 20 minutes). Proteins were cross-linked to DNA (1% formaldehyde, 10 minutes). Cells were washed with PBS, scraped, collected, lysed [1% SDS, 10 mmol/L EDTA, 50 mmol/L Tris (pH 7.9), 1 mmol/L DTT, protease inhibitors (Roche)], and incubated on ice for 45 minutes. Samples were sonicated thrice, and chromatin precleared with protein A beads for 2 hours. Antibodies (3.5 μg) were added and mixtures incubated overnight at 4°C. Protein A beads were added and mixed (2 hours, 4°C). Beads were washed twice with low-salt buffer (150 mmol/L NaCl, 0.5% deoxycholate, 0.1% Nonidet P-40, 1 mmol/L EDTA, 50 mmol/L Tris-HCl), twice with high salt buffer (low salt buffer + 500 mmol/L NaCl), and twice with Tris-EDTA buffer. Cross-link was then reversed (100 mmol/L $NaHCO_3$, 1% SDS, overnight 65°C), proteins digested with proteinase K (1 hour), and DNA purified using the nucleotide extraction kit (Qiagen). The DR-2–containing region of the *caspase 9* gene was amplified by PCR using the primers 5′-tgccatgtctaccacggcacagg-3′ (forward) and 5′-tgcaccatgcctggctagtt-3′ (reverse).

Affymetrix expression array. MCF-7 cells were transduced with Ad-0 virus at an MOI of 500 for 18 hours, followed by a 4-hour treatment with 50 nmol/L RA or vehicle. Total RNA was prepared from triplicate cell cultures using RNeasy (Qiagen). Sample processing and analyses, including cDNA synthesis, cRNA synthesis, and labeling, and array applications were done at the University of Rochester Functional Genomics Center. RNA quality was assessed using the Agilent Bioanalyzer 2100 and spectrophotometric analysis before cDNA synthesis. Five micrograms of total RNA from each sample were used to generate cDNA, and 1 μg product was used in an *in vitro* transcription reaction containing biotinylated UTP and CTP. Twenty micrograms of full-length cRNA were fragmented and analyzed on Affymetrix U133A/B high-density oligonucleotide array. Arrays were hybridized, stained, and washed using the Affymetrix fluidics module. Detection and quantitation of target hybridization were done with a GeneArray Scanner 3000 (Affymetrix, Santa Clara, CA). Iobion's Gene traffic was used for Robust Multi-Chip Analysis and clustering of genes with similar activity by summary function. An unpaired *t* test giving *P* values of <0.1 were defined as significantly changed.

Electrophoretic mobility shift assay. DNA probes were generated by restriction enzyme digestion of the luciferase reporter construct containing the DR-2 region of the caspase 9 intron 2. Oligonucleotides were end-labeled with [^{32}P]dCTP by filling in with Klenow fragments, and free nucleotides were removed with the Qiagen nucleotide removal kit. DNA (10,000 cpm, 16 fmol) was incubated with 1.25 pmol of receptor for 20 minutes. Cold competitor DNA was composed of 45 bp encompassing the DR-2 intron element, or a corresponding mutant DNA (DR-2, TCTGCCGGTCTGCC). Protein-DNA complexes were resolved by electrophoresis on 5% polyacrylamide gels and visualized by autoradiography.

Results

Retinoic acid induces apoptosis in MCF-7 cells. MCF-7 cells were treated with varying concentrations of RA for 5 days and analyzed by flow cytometry. The analyses (Fig. 1) showed that RA triggered DNA fragmentation, reflected by a RA dose-responsive increase of the fraction of cells in sub-G_1 population. A 3-fold increase in apoptosis was observed at the highest RA concentration used (0.6 μmol/L, Fig. 1*B*). In contrast, RA had little effect on the distribution of cells between different cell cycle phases (Fig. 1*C*). Hence, under this experimental regimen, inhibition of MCF-7 cell growth by RA is mediated primarily by induction of apoptosis.

Retinoic acid up-regulates expression of proapoptotic genes in MCF-7 cells. We thus sought to identify target genes that may mediate the proapoptotic activity of RA. To this end, we carried

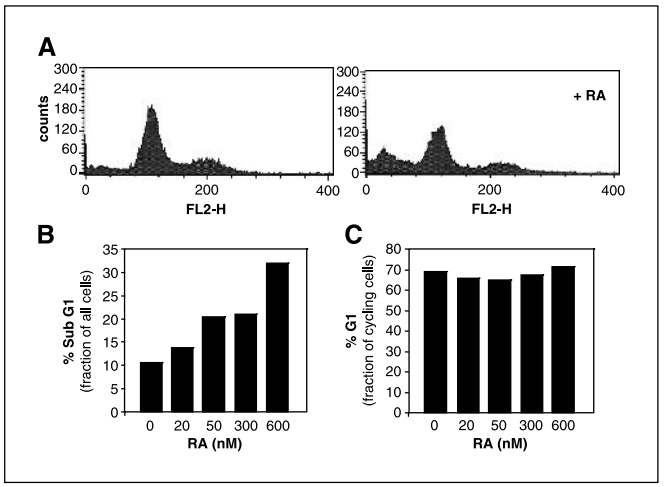

Figure 1. RA induces apoptosis and does not affect cell cycle distribution in MCF-7 cells. *A,* representative histogram of FACS analysis of MCF-7 cells untreated (*left*) or treated (*right*) with 600 nmol/L RA for 5 days. *B,* fraction of cells in sub-G_1 following a 5-day treatment with RA. *C,* fraction of cycling cells in G_1 phase following a 5-day treatment with RA.

out an expression array analysis. MCF-7 cells were treated with 50 nmol/L RA or vehicle for 4 hours and total RNA was isolated. Probes were generated, hybridized to Affymetrix human U133 A/B arrays, which allows for monitoring the expression of more than 40,000 genes and ESTs, and differences in gene expression profiles between RA-treated and untreated cells were analyzed. Three replicates were analyzed for each condition and changes in genes that were observed in all replicates and that displayed *P* values of <0.1 (unpaired *t* test) were considered to be significant. A total of 825 genes were found to be up-regulated by at least 1.23-fold on RA treatment, with the highest fold (×18.25) observed for the well-characterized RA-responsive gene *Cyp26a* (25). RA-induced genes were clustered by similar biological functions using the GeneTraffic software system (Iobion). One such cluster was found to encompass several genes that are known to be involved in apoptotic responses (Table 1). The observations that the expression of several such genes was up-regulated by RA and the relatively small magnitude of the responses suggest that induction of apoptosis by RA in MCF-7 cells may result from concerted, cumulative effects on multiple pathways. Notably, however, two genes in this cluster encode for proteins that are known to directly mediate apoptosis (i.e., caspase 7 and caspase 9). Regulation of expression of RA-responsive genes may be exerted directly (i.e., mediated by an RARE). Alternatively, responses may reflect secondary events involving RAR control of immediate target genes, which, in turn, are involved in downstream events leading to the observed modulation. Hence, an important question that arises is which of the RA-controlled proapoptotic genes in MCF-7 cells comprise direct targets for RAR. This question is particularly pertinent considering the present paucity of information on the mechanisms by which RA exerts its anticarcinogenic activities. To begin to address this issue, we examined whether either caspase 7 or caspase 9 is under direct RAR control.

***Caspase 9* is a direct target for retinoic acid receptor, *caspase 7* is not.** To validate the array data, quantitative real-time PCR was carried out. MCF-7 cells were treated for 4 hours with RA (50 nmol/L) or vehicle, and quantitative real-time PCR was used to compare the expression levels of mRNA for caspase 9 and caspase 7 in RA-treated versus untreated cells (Fig. 2). In good agreement with the Affymetrix array data (Table 1), the expression levels of mRNA for both caspase 9 and caspase 7 increased by about 60% in response to RA treatment. To determine whether these genes are direct targets for transcriptional regulation by RA, the effect of the protein synthesis inhibitor cycloheximide on their induction by RA was studied. Cycloheximide treatment will abolish secondary events that require *de novo* protein synthesis but will not affect direct transcriptional responses. The analyses showed that inhibition of protein synthesis completely abolished the RA response of caspase-7 expression (Fig. 2*A*). In contrast, caspase 9 mRNA was up-regulated by RA regardless of the presence of cycloheximide (Fig. 2*B*). Hence, whereas the effect of RA on caspase-7 expression is a secondary response, *caspase 9* is likely to be a direct target for RAR signaling.

A functional retinoic acid response element is present in the second intron of the caspase 9 gene. Consensus RAREs are composed of two direct repeats of the sequence PuG(G/T)TCA spaced by either 2 bp (DR-2) or 5 bp (DR-5). We used two programs, TransFac (26)[1] and TESS (27),[2] to screen the human *caspase 9* gene and adjacent regulatory sequences for potential RAREs. Within a stretch of 8 kb upstream of the *caspase 9* start site (1p36.3), a potential RARE composed of the noncanonical DR-2 sequence AGGTCAgcAGTTCG was found at position −1690. This element, along with 38 bp of flanking sequences on both sides, was cloned into a luciferase reporter vector and its functionality was examined by transactivation assays carried out in MCF-7 cells. The expression of the reporter did not respond to RA (Fig. 2*C*), suggesting that the element does not function as an RARE. An additional potential RARE, composed of the consensus DR-2 sequence AGGTCAggAGTTCA, was found in the second intron of the *caspase 9* gene, 9,461 bp downstream of the start site. This RARE and 45 bp of flanking sequences on each side were cloned into a luciferase reporter, which was used in transactivation assays. The data (Fig. 2*D*) showed a dose-responsive activation of reporter expression by RA, suggesting that the element indeed comprises a functional RARE. To verify that the element can bind RAR-RXR heterodimers, electrophoretic mobility shift assays were carried out. RARα and RXRα lacking their amino terminal A/B domains (RARαΔAB and RXRαΔAB) were expressed in *E. coli*, purified, and examined for binding to a 90 bp oligonucleotide harboring the putative response element and its flanking sequences. The data (Fig. 2*E*) showed that RAR-RXR heterodimers tightly and specifically associate with the DR-2 element of the second intron of *caspase 9*. Finally, to examine whether the element is occupied by RXR-RAR heterodimers in a living cell, chromatin immunoprecipitation assays were carried out. Proteins were cross-linked to chromatin in MCF-7 cells and immunoprecipitated using antibodies for RAR or RXR, or nonspecific immunoglobulin G (IgG). Precipitates were sonicated, the cross-link reversed, DNA isolated, and a 250 bp region surrounding the DR-2 in the second intron of *caspase 9* amplified by PCR. The data (Fig. 2*F*) showed that antibodies against either RAR or RXR specifically precipitated the intron DR-2 sequence, demonstrating that the element is occupied by the heterodimers

[1] http://www.gene-regulation.com.
[2] http://www.cbil.upenn.edu/tess.

Table 1. Proapoptotic genes induced by RA in MCF-7 cells

Gene	Fold change	*P*	Accession no.
IFN regulatory factor 1	1.66	0.012061	NM_002198
TNF receptor superfamily, member 1A	1.65	3.99e−06	NM_001065
Caspase 7, apoptosis-related cysteine protease	1.39	0.023309	NM_001227
Caspase 9, apoptosis-related cysteine protease	1.37	0.012061	U60521
TIA1 cytotoxic granule-associated RNA binding protein	1.33	0.027382	AL046419
Small inducible cytokine subfamily E, member 1	1.32	0.067476	BF589679

NOTE: MCF-7 cells were treated with vehicle or RA (50 nmol/L) for 4 hours. The mRNA expression profiles of treated versus untreated cells were compared by Affymetrix array analyses (see Materials and Methods). Shown are RA-induced changes in mRNA levels of genes involved in apoptosis.

in cells. Taken together, the observations establish that caspase 9 is a direct target for RAR signaling, and that the RARE responsible for this response is likely to be a DR-2 element located in the second intron of the gene.

Cellular retinoic acid-binding protein II augments the induction of caspase-9 expression by retinoic acid. We previously showed that the RA-binding protein CRABP-II facilitates the delivery of RA to RAR, thereby enhancing the transcriptional activity of the receptor, at least in the context of a reporter construct driven by a consensus RARE. These observations suggest that RA-induced up-regulation of direct RAR target genes will be augmented by CRABP-II. It may also be suggested that, due to dilution of the effect in subsequent steps, RA responses mediated by secondary events may be less amenable to modulation by the binding protein. The finding that *caspase 9* comprises a direct target for RAR in MCF-7 cells whereas *caspase 7* is an indirect responder provides an opportunity to examine the activity of CRABP-II in the context of an endogenous gene driven by a native promoter, and to test the effect of the binding protein on two genes that are differentially regulated by RA. We thus investigated the effect of overexpression of CRABP-II in MCF-7 cells on RA-induced up-regulation of the two caspases. CRABP-II was ectopically expressed using an adenovirus harboring CRABP-II cDNA (Fig. 3*A*). Initial experiments verified that viral infection resulted in a pronounced CRABP-II overexpression, which could be observed 24 hours following infection and maintained for longer than 6 days (Fig. 3*B*). MCF-7 cells were infected with Ad-CRABP-II for 24 hours, treated with RA for 4 hours, and the expression of caspase-7 and caspase-9 mRNA was examined by quantitative real-time PCR. The data (Fig. 3*C*)

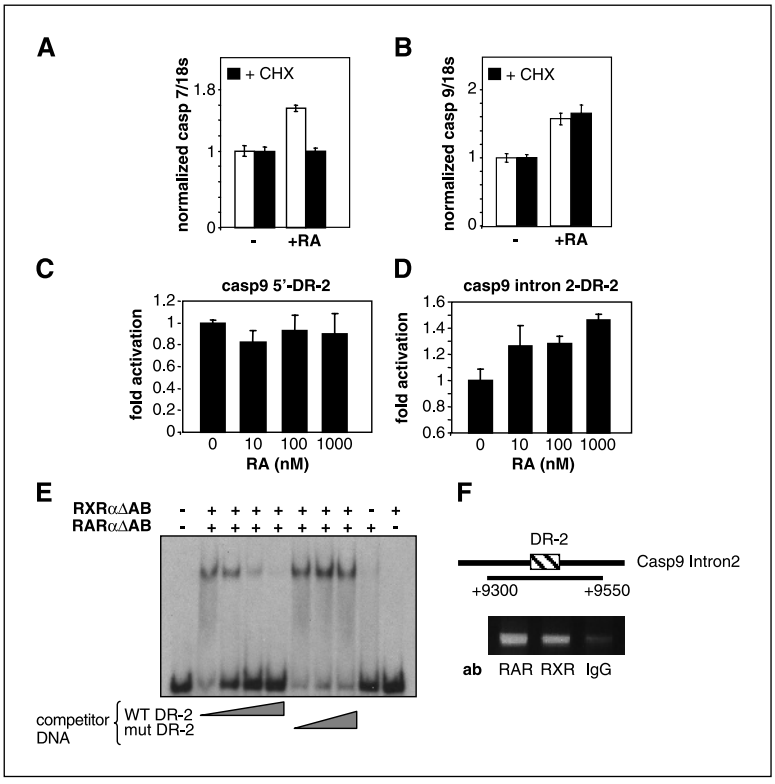

Figure 2. *Caspase 9* is a direct target for RAR and possesses a functional RARE in its second intron. MCF-7 cells were treated with vehicle or with 50 nmol/L RA for 4 hours in the absence or presence of pretreatment with cycloheximide (*CHX*). Expression levels of mRNA for caspase 7 (*A*) or caspase 9 (*B*) were measured by quantitative real-time PCR and normalized to 18s mRNA. Fold induction by RA is shown. *Columns,* mean ($n = 4$); *bars,* SD. *C* and *D,* transactivation assays using MCF-7 cells transfected with luciferase reporter constructs driven by the DR-2 of the promoter of *caspase 9* (*C*) or by the DR-2 element of the *caspase 9* intron 2 (*D*). *Columns,* mean ($n = 3$); *bars,* SD. *E,* electrophoretic mobility shift assay analysis of the interactions of RXR-RAR complexes with the intron DR-2 element. Bacterially expressed RARαΔAB and RXRαΔAB were mixed with ^{32}P-labeled oligonucleotide containing the DR-2 element of intron 2. Mixtures were resolved by nondenaturing PAGE and visualized by autoradiography. Competitor DNA (1.5, 4.6, or 9.3 pmol) containing wild-type or mutant DR-2 was added as indicated. *F,* chromatin immunoprecipitation analysis of the intron 2 DR-2 in MCF-7 cells. Immunoprecipitations were carried out using nonspecific IgG or antibodies against RAR or RXR. The region of intron 2 of the *caspase 9* gene containing the putative RARE was amplified by PCR (see Materials and Methods).

showed that CRABP-II overexpression augmented the RA-induced up-regulation of expression of the direct RAR target gene *caspase 9* by about 20%, a modest but clearly significant effect which about doubled the RA induction of the gene in this experiment. CRABP-II expression had little effect on the RAR indirect target, *caspase 7* (Fig. 3*D*).

Retinoic acid and cellular retinoic acid-binding protein II induce cleavage of caspases 7 and 9. One of the hallmarks of the apoptotic response is the activation of caspases which results from cleavage of the inactive zymogens to their active forms. To directly examine whether up-regulation of the expression levels of caspases 7 and 9 by RA (Fig. 2) is accompanied by their activation, cells were treated with RA and cell lysates were probed for the presence of the activated forms of the proteases using antibodies directed against cleaved caspase 7 or caspase 9. The cleaved product of caspase 7 was undetectable in untreated MCF-7 cells, and its level was slightly increased in response to RA treatment (Fig. 4*A*). The level of cleaved caspase 9 markedly increased on RA treatment and did so in a dose-responsive fashion (Fig. 4*B*). Strikingly, overexpression of CRABP-II induced cleavage of both caspase 7 and caspase 9 even in the absence of RA. In the presence of RA, CRABP-II markedly augmented the ability of RA to trigger caspase cleavage (Fig. 4*A-C*). Especially notable is that, whereas RA had only a small effect on the cleavage of caspase 7 on its own, it dramatically enhanced the activation of this caspase on CRABP-II overexpression, and did so in a dose-dependent manner.

Cellular retinoic acid-binding protein II up-regulates the expression of Apaf1 in the absence of retinoic acid. The observation that CRABP-II expression leads to activation of both caspase 7 and caspase 9 even in the absence of RA suggests that, in addition to its ability to augment the RA-induced activation of RAR, CRABP-II may display a RA-independent activity. These findings further suggest that this activity allows the binding protein to modulate either the expression level or the activity of component(s) in the pathway that leads to caspase cleavage. Activation of caspases on apoptotic signaling is triggered by the release of mitochondrial cytochrome *c*, which, in turn, binds the cytosolic adaptor protein Apaf1 to form a multiprotein complex called the apoptosome. The apoptosome, with Apaf1 at its core, cleaves procaspase 9, which then activates executioner caspases, caspase 7, and caspase 3, to propagate the apoptotic response (28). Considering the central role of Apaf1 in caspase activation, we examined whether CRABP-II may modulate Apaf1 expression. MCF-7 cells were infected with either Ad-0 or Ad-CRABP-II for 24 hours, and quantitative real-time PCR was used to measure Apaf1 mRNA levels. The data (Fig. 5*A*) showed that overexpression of CRABP-II per se resulted in a 2-fold increase in the level of Apaf1 mRNA. It could be argued that this effect may reflect a CRABP-II–enhanced activity of residual RA present in the cells rather than a RA-independent activity of the binding protein. This is unlikely both because the cells were depleted of vitamin A and RA stores by culturing in media containing charcoal-treated serum and because MCF-7 cells are severely impaired in their ability to synthesize RA (29). Nevertheless, we examined whether Apaf1 comprises a RA-responsive gene. MCF-7 cells were treated with 50 nmol/L RA for 24 hours and Apaf1 mRNA level was measured by quantitative real-time PCR. RA treatment had no effect on Apaf1 mRNA expression (Fig. 5*B*), demonstrating that it is not a target for RA signaling and supporting the conclusion that CRABP-II modulates the expression of this gene by a RA-independent mechanism.

Cellular retinoic acid-binding protein II augments retinoic acid–induced apoptosis in MCF-7 cells. The finding that CRABP-II augments the RA-induced expression of caspase 9, and that it cooperates with RA in enhancing the activation of both caspase 7 and caspase 9, suggests that the binding protein is closely involved in apoptosis initiated in MCF-7 cells on RA

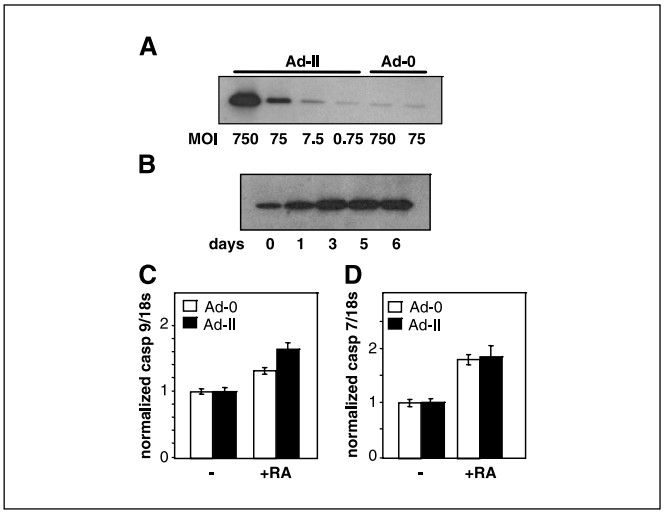

Figure 3. CRABP-II augments RA-induced up-regulation of caspase-9 expression. *A* and *B,* overexpression of CRABP-II by adenoviral transduction of MCF-7 cells. Cells were transduced with either an empty virus (Ad-0) or adenovirus harboring the CRABP-II cDNA (Ad-II), and protein expression was monitored by Western blots. *A,* cells were treated with the indicated amounts of Ad-0 or Ad-II for 24 hours. *B,* cells were treated with 500 MOI Ad-II for the indicated times. *C* and *D,* effect of CRABP-II overexpression on RA-induced up-regulation of mRNA for caspase 9 (*C*) and caspase 7 (*D*). MCF-7 cells were infected with Ad-0 or Ad-II for 24 hours and then treated with vehicle or 50 nmol/L RA for 4 hours. Expression levels of mRNA for the caspases were measured by quantitative real-time PCR. *Columns,* mean ($n = 4$); *bars,* SD.

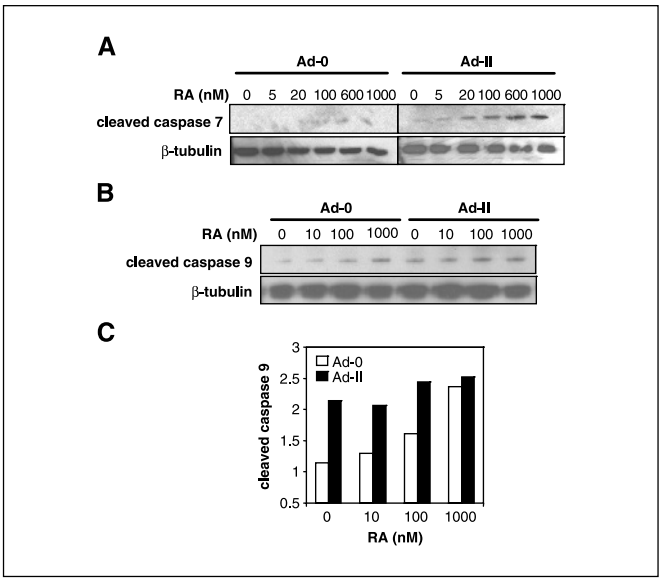

Figure 4. RA and CRABP-II induce caspase cleavage. MCF-7 cells were infected with Ad-0 or Ad-CRABP-II (Ad-II) for 24 hours and then treated with RA for 5 days, replenishing the ligand every 48 hours. Cleavage of caspase 7 (*A*) or caspase 9 (*B*) was monitored by Western blot analyses using antibodies against the cleaved forms of the respective caspases. *C,* quantitation of data presented in (*B*).

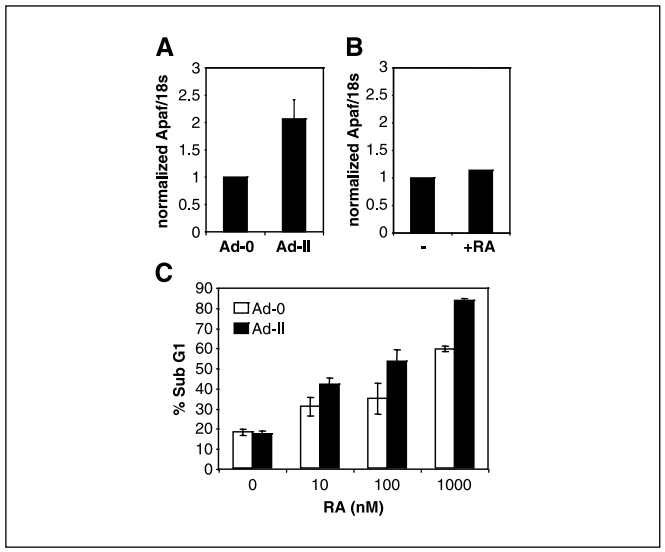

Figure 5. CRABP-II up-regulates Apaf1 expression and amplifies RA-induced DNA fragmentation. *A,* MCF-7 cells were infected with Ad-0 or Ad-CRABP-II for 24 hours. Apaf1 and 18s mRNA levels were measured by quantitative real-time PCR. Values [*columns,* mean (n = 3); *bars,* SD] were normalized to 18s mRNA levels and fold induction on CRABP-II overexpression is shown. *B,* MCF-7 cells were treated with 50 nmol/L RA for 24 hours. Apaf1 and 18s mRNA levels were measured by quantitative real-time PCR. Values, normalized to 18s mRNA levels, represent the mean of two independent experiments which varied by 10% (−RA) and 12% (+RA). *C,* MCF-7 cells were infected with Ad-0 or Ad-CRABP-II for 24 hours before treatment with the indicated amounts of RA for 7 days. Cells were analyzed by FACS to measure the fraction of cells in the sub-G_1 population. *Columns,* mean (n = 3); *bars,* SD.

treatment. To directly examine this possibility, flow cytometry was used to monitor the effect of CRABP-II expression on RA-induced DNA fragmentation. MCF-7 cells were infected with Ad-CRABP-II or Ad-0 for 18 hours and then treated with varying concentrations of RA for 7 days. Nuclei were isolated, stained with propidium iodide, and the fraction of cells containing fragmented DNA (sub-G_1) determined. The data (Fig. 5*C*) showed that overexpression of CRABP-II indeed markedly enhanced RA-induced apoptosis over a wide range of ligand concentrations.

Discussion

RA displays pronounced anticarcinogenic activities in several types of cancer but the exact mechanisms that underlie these activities remain incompletely understood. The present work was undertaken to obtain insights into molecular events through which RA inhibits the growth of MCF-7 mammary carcinoma cells. Of special interest with regard to this issue is the involvement of the two proteins that mediate the transcriptional activity of RA, the nuclear hormone receptor RAR and the RA binding protein CRABP-II, in the antiproliferative activity of their ligand. The data presented above show that a 5- to 7-day treatment of MCF-7 with RA induced pronounced apoptosis but had little effect on cell cycle distribution (Fig. 1). These findings are consistent with our previous observations that breast tumor suppression by RA and CRABP-II in the MMTV-*neu* mouse cancer model results from induction of apoptosis and not from effects on cell cycle progression (23).

Correspondingly, examination of the effect of RA on the gene expression profile of MCF-7 cells revealed that several proapoptotic genes are up-regulated on a 4-hour exposure to RA (Table 1). At this short period of treatment, it can be expected that at least some of the responding genes comprise direct targets for RAR. Additionally, due to its ability to enhance the transcriptional activity of RAR, it may be predicted that CRABP-II will augment the RA response of genes that are under direct RAR control. We show that *caspase 9* is indeed a direct target for RAR (Fig. 2) and that the induction increases on CRABP-II overexpression (Fig. 3). These observations comprise the first demonstration that CRABP-II enhances the RA-induced, RAR-mediated activation of an endogenous gene.

The conclusion that caspase 9 is under the direct control of RAR prompted us to search for the RARE through which the regulation is exerted. We show that the second intron of the caspase 9 gene harbors a DR-2 RARE, that the element specifically binds RXR-RAR heterodimers, that it mediates RA-induced transcriptional activation of a luciferase reporter, and that it is occupied by RXR-RAR heterodimers in MCF-7 cells (Fig. 2). The identification of the caspase-9 RARE in an intron sequence contributes to the growing body of evidence that regulatory elements are not confined to upstream promoter regions of target genes. Additional examples include the identification of clusters of glucocorticoid response elements in introns 1 and 2 of Granzyme A and FKBP5, respectively (30), and of a PPAR response element within intron 3 of the *FIAF* gene (31). This notion is further supported by the recent report that only 22% of binding sites for Sp1, c-myc, and p53 on chromosomes 21 and 22 are located at the 5′ termini of protein-coding genes, with the remainder positioned within or immediately 3′ to well-characterized genes (32). An interesting question that arises from these observations relates to the temporal occupation of intron response elements following gene activation. Presumably, the transcription factor will be effectively displaced from the element by the traveling polymerase.

In addition to caspase 9, RA treatment of MCF-7 cells up-regulated the expression of mRNA for caspase 7. However, unlike caspase 9, the induction of caspase 7 by RA was abolished on treatment with cycloheximide (Fig. 2), indicating that this gene is an indirect target for RA signaling. Unlike caspase 9 also, CRABP-II did not augment RA-induced up-regulation of caspase 7 (Fig. 3). As the effect of RA on *caspase 7* is likely to be mediated by another protein which, in turn, is under RA control, the ineffectiveness of CRABP-II in the context of this gene may result from dilution of the binding protein effect in secondary steps. Alternatively, it is possible that the protein that directly regulates *caspase-7* expression is only needed at low levels, rendering CRABP-II function unnecessary.

Overexpression of caspases and other components of the apoptotic response has been shown to trigger apoptosis or to increase the susceptibility of cells to apoptosis-inducing agents (33–35). In keratinocytes, RA treatment was reported to up-regulate the expression of several caspases. The mechanism by which RA increases caspase expression in these cells remains unknown, but it has been shown that although their overexpression does not induce apoptosis by itself, it sensitizes the cells to apoptosis induced by UVB irradiation and by doxorubicin (36). We show here that in MCF-7 cells, RA increases the expression of several proapoptotic genes and leads to pronounced apoptosis without the need for additional agents. It is likely that the observed RA-induced apoptosis stems from a cumulative effect of the increased expression of multiple proapoptotic genes. It is worth noting with regard to this that it has been reported that RA treatment of MCF-7 cells leads to activation of Bax and to the release of mitochondrial cytochrome *c* (37). The

mechanism by which RA affects Bax remains to be clarified, but its activation, together with the increased levels of the proapoptotic factors identified here, may coalesce to trigger and execute programmed cell death in MCF-7 cells in response to RA.

Perhaps the most surprising findings of this study are that CRABP-II, a protein that is known to cooperate with RAR in regulating the transcriptional activity of RA (19, 21, 22), also displays RA-independent activities. The data presented here show that, on its own, CRABP-II up-regulates the expression of Apaf1 and triggers the activation of caspase 7 and caspase 9. These observations suggest that the tumor-suppressor activities of CRABP-II (23) may stem from two separate biological functions: the known role of this protein in the direct delivery of RA to RAR and another, RA-independent, activity. The nature of this additional function for CRABP-II remains to be delineated. Finally, we note that although CRABP-II displays proapoptotic activities, it does not induce apoptosis on its own. As the induction of cell death is likely to depend on certain thresholds of proapoptotic components, the data suggest that CRABP-II sensitizes cells to apoptosis (i.e., it cooperates with other apoptotic agents to enable a more efficient induction of cell death).

Acknowledgments

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